IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 1693
Helen H. Hobbs et al.	Examiner: Christopher J. Nichols
Application No.: 09/989,981	Technology Center/Art Unit: 1647
Filed: November 20, 2001	DECLARATION OF HELEN HOBBS UNDER 37 CFR § 1.131
For: ABCG5 AND ABCG8: COMPOSITIONS AND METHODS OF USE	
Customer No.: 20350	

Commissioner for Patents
Box 1450
Alexandria, VA 22313-1450

Sir:

I, Helen Hobbs, was at the time of the invention employed by the University of Texas System, which is a co-assignee of the above-referenced patent application. I am, with co-inventors Bei Shan, Robert Barnes and Hui Tian, one of the named and true inventors of the subject matter disclosed and claimed in the above-referenced patent application.

I and co-inventors Bei Shan, Robert Barnes and Hui Tian conceived of and reduced to practice the claimed invention in the United States prior to October 27, 2000. The attached Exhibits A and B provide evidence of the conception of

1

Helen H. Hobbs, *et al.*   <u>PATENT</u>
Application No.: 09/989,981
Page 2

the invention and its reduction to practice. Exhibits A and B, with dates redacted therefrom, were written prior to October 27, 2000.

Exhibit A is a manuscript submission, that was prepared by the inventors, including me, and submitted to *Science* magazine prior to October 27, 2000. The manuscript was ultimately published as Berge, *et al.* "Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters," *Science* 290:1771 (December 1, 2000). The manuscript submission describes conception and reduction to practice of mouse and human ABCG5 and ABCG8 (*see*, Figure 1 on page 18 and the Figure 2 legend on pages 16-17).

Exhibit B is a copy of an email sent from me to the attorney of record before October 27, 2000 and the human ABCG5 and ABCG8 cDNA sequences that were attached. These sequences are designated as SEQ ID NO:5 and SEQ ID NO:7 in the above-referenced application. The date the email was sent has been redacted.

I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: ___2/5/04___   _____
   Helen H. Hobbs, Ph.D.

60123755 v1

Accumulation of Dietary Cholesterol in Sitosterolemia Caused by Mutations in Adjacent ABC Transporters

Knut E. Berge,[1][*] Hui Tian,[3][*] Gregory A. Graf,[1] Liqing Yu,[1] Nick V. Grishin,[2] Joshua Schwartz,[3] Peter Kwiterovich,[4] Bei Shan,[3] Robert Barnes,[1] Helen H. Hobbs[1][**]

[1]Department of Molecular Genetics and McDermott Center for Human Growth and Development and [2]Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75390-9046, [3]Tularik Inc., Two Corporate Drive, South San Francisco, CA 94080 and [4]Department of Pediatrics, Johns Hopkins University, Baltimore, MD

* These authors contributed equally to the work

**To whom correspondence should be addressed: Helen.Hobbs@UTSouthwestern.edu

1
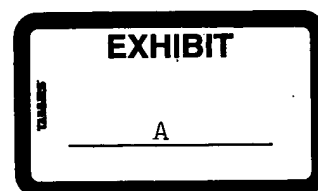
ABSTRACT

In normal humans, acute changes in cholesterol intake produce modest changes in plasma cholesterol levels. A striking exception occurs in sitosterolemia, an autosomal recessive disorder characterized by increased intestinal absorption and decreased biliary excretion of dietary sterols, hypercholesterolemia and premature coronary atherosclerosis. We have identified mutations in two adjacent, oppositely-oriented genes in sitosterolemia. Both genes (*ABCG5* and *ABCG8*) encode new members of the ATP-binding cassette (ABC) transporter family. The two genes are expressed at highest levels in liver and intestine and are up-regulated coordinately by cholesterol feeding. The data suggest that ABCG5 and ABCG8 normally cooperate to limit intestinal absorption and promote biliary excretion of sterols, and that mutated forms of these transporters predispose to sterol accumulation and atherosclerosis.

In humans the intestine presents a barrier that prevents the absorption of plant sterols and partially blocks the absorption of cholesterol. This barrier is disrupted in the rare autosomal recessive disorder, sitosterolemia (1). Sitosterolemic patients hyperabsorb the plant sterols such as sitosterol, which provide the identifying feature of this disease (1-3). These patients also hyperabsorb cholesterol and are usually hypercholesterolemic, resulting in the development of xanthomas (cholesterol deposits in skin and tendons) and premature coronary artery disease (2,3). Unlike other forms of hyperlipidemia,

sitosterolemic subjects respond to restriction in dietary cholesterol and to bile acid resin treatment with dramatic reductions in plasma cholesterol levels (2-5).

Patients with sitosterolemia have markedly elevated (>30-fold) plasma levels of plant sterols (sitosterol, stigmasterol and campesterol) as well as other neutral sterols with modified side chains (1,6,7). Normal humans absorb only ~5% of the 200 to 300 mg of plant sterols consumed each day (8,9). Almost all of the absorbed sitosterol is quickly secreted into the bile so that only trace amounts of sitosterol and other plant sterols remain in the blood (8,9). In contrast, subjects with sitosterolemia absorb between 15 and 60% of ingested sitosterol, and they excrete only a fraction into the bile (1-4). The liver secretes sitosterol into the bloodstream where it is transported as a constituent of low density and high density lipoprotein particles (1). With the exception of the brain, the relative proportion of sterol represented by sitosterol in tissues matches that in plasma (10-25%) (10). Hyperabsorption and inefficient secretion is not limited to plant sterols. Sitosterolemic subjects absorb a higher fraction of dietary cholesterol than normal subjects, and they secrete less cholesterol into the bile (1-4). Taken together, the genetic and metabolic data indicate that sitosterolemic patients lack a gene product that normally limits the absorption and accelerates the biliary excretion of sterols (2,3).

The molecular mechanisms that limit sterol absorption are poorly understood but clues have emerged recently from studies of the orphan nuclear hormone receptor LXR (11). Mice with a targeted disruption of the LXR gene show enhanced cholesterol absorption (12). Conversely, mice treated with an LXR agonist have a marked decrease in cholesterol absorption and a corresponding increase in the intestinal expression of

3

mRNA encoding the ATP binding cassette protein (ABC) 1, a membrane-associated protein that has been implicated in the transport of cholesterol (11,13). We hypothesized that sitosterolemic patients might have defects in other genes that limit cholesterol absorption and that the expression of these genes would be regulated by LXR. To test this idea we used DNA microarrays to search for mRNAs that are induced by the LXR agonist T091317 in mouse liver and intestine (11,14). A transcript corresponding to a murine EST (AA237916) was induced ~2.5-fold in the livers and intestines of treated mice. This EST resembled three *Drosophila* genes that encode ABC half-transporters (*brown*, *scarlet* and *white*) expressed in the pigmentary cells of the eye (15-17). These ABC half-transporters contain six membrane spanning domains and form two types of heterodimers that transport guanine (*brown/white*) or tryptophan (*scarlet/white*). Since a human homolog of *white* (*ABCG1*) is implicated in cellular cholesterol efflux from macrophages (18,19), we reasoned that the LXR-induced protein encoded by AA237916 might be involved in sterol trafficking in liver and intestine, and hence this gene was a candidate for the defect in sitosterolemia.

A full-length cDNA corresponding to AA237916 was isolated from a mouse liver cDNA library (Origene), and this sequence was used to identify a human ortholog in the GENBANK EST database (T86384). A full-length human sequence was obtained by iterative EST database searches and by cloning from human liver cDNA libraries (Origene and Clontech). The human cDNA predicts a 651 amino acid protein (Fig. 1C) that shares 82% sequence identity with the mouse sequence (data not shown). Following the standard system of nomenclature in the ABC transporter field, we have named this

4

protein *ABCG5*. The amino terminal half of *ABCG5* contains the ATP-binding motifs (Walker A and B motifs) and an ABC transporter signature motif (C motif), while the carboxyl terminal region is predicted to contain six transmembrane (TM) segments (Fig. 1B) (17,20,21). A human EST clone (Unigene T93792) from *ABCG5* mapped to chromosome 2p21 between markers D2S177 and D2S119 and the map position was confirmed using a radiation hybrid panel (22). Patel and colleagues previously mapped the human sitosterolemia gene to this same region of chromosome 2 in ten independent sitosterolemic families (23).

The structure of the human *ABCG5* gene was characterized by analysis of a bacterial artificial chromosome (BAC) clone that contained the entire gene (Fig. 1A) (24). The gene spans ~28 kb and has 13 exons and 12 introns. The coding sequences and consensus splicing sequences were amplified from genomic DNA by PCR and sequenced in nine unrelated subjects with sitosterolemia (Table 1). A sitosterolemic patient from Hong Kong (proband 9) was heterozygous for a transition mutation (CGA to TGA) in codon 408 that introduced a premature stop codon between TM1 and TM2. This mutation was not present in 65 normolipidemic individuals, including 40 Chinese subjects. No other potential disease-causing mutations were identified in *ABCG5*. A transversion in codon 604 that substituted a glutamic acid for glutamine (Q604E) in the loop between TM5 and TM6 was found in five sitosterolemic probands but was also present in 23% of the alleles from normolipidemic Caucasians (n=50).

Genes encoding members of the ABC transporter family are often clustered in the genome (25). Since only a single *ABCG5* mutation was identified in our collection of

nine sitosterolemic probands, we searched the public and Celera genome sequences for other ABC transporters adjacent to *ABCG5*. An EST (T84531) that shared weak homology with the *Drosophila white* gene was identified and expanded using exons predicted by the computer program GENSCAN (26). Eleven of the 13 exons of the new gene, which we name *ABCG8*, were identified in the databases and the remaining two exons were identified by sequencing PCR-amplified cDNAs from human hepatic polyA$^+$ mRNA. *ABCG8* shares ~28% amino acid identity with *ABCG5* (Fig. 1C). Its sequence is most similar to *ABCG1*, which resembles the *Drosophila white* gene (16).

The translational start sites of *ABCG5* and *ABCG8* are separated by only 374 basepairs and the two genes are arranged in a head to head orientation (Fig. 1A). Both genes contain a translation initiation codon with an upstream in-frame stop codon. The close proximity and opposite orientation of *ABCG5* and *ABCG8* suggest that the two genes have a bi-directional promoter and share common transcriptional and regulatory elements (27,28). No obvious LXR response element was identified in the limited amount of sequence available at this time. Other gene pairs with bi-directional promoters form functional complexes (28), as may be the case for *ABCG5* and *ABCG8*.

The predicted intron-exon boundaries of human *ABCG8* were confirmed by DNA sequencing. The single strand conformation polymorphism technique was used to screen the exons and flanking intron sequences of *ABCG8* in the nine sitosterolemic subjects (Table 1) (29,30). DNA sequencing of abnormally-migrating fragments revealed six different mutations (Table 1 and Fig. 1B). The first patient to be described with sitosterolemia (proband 1) was homozygous for a nonsense mutation (c.1083G>A) in

exon 7 (Fig. 1B) that introduced a premature termination signal codon at codon 361, terminating the protein prior to TM1 (1). Three other unrelated Caucasian sitosterolemic subjects (probands 3, 5 and 8) were heterozygous for the same mutation (3,31,32). One of these probands (proband 5) were originally given the diagnosis of pseudohomozygous familial hypercholesterolemia, an autosomal recessive disorder characterized by hypercholesteromia, tendon xanthomas and exquisite sensitivity to dietary cholesterol (5,32). Many of the patients originally diagnosed with pseudohomozygous FH were subsequently found to have sitosterolemia, as was the case with this patient and proband 6 (5,32). Proband 3 was heterozygous for another nonsense mutation in exon 13 that introduced a stop codon 15 residues from the carboxyl terminus of *ABCG8*. The resulting protein would lack part of the last predicted transmembrane domain, and the short cytoplasmic domain, which contains a cluster of positively charged residues that may be important in positioning these proteins in the membrane (33).

Two missense mutations identified in *ABCG8* produced nonconservative amino acid changes in residues that are conserved between the humans and mouse proteins (data not shown) as well as in *ABCG5*. One Chinese patient (proband 4) was heterozygous for a missense mutation in exon 6 in codon 263 (R263Q). An Amish subject with sitosterolemia was homozygous for a missense mutation (G574R) in a residue that is conserved in mouse and human *ABCG8*. Genomic DNA was available from an additional three of the four living affected family members in this large Amish pedigree (34,35) and these individuals were homozygous for this same missense mutation (data not shown). A third nonconservative missense mutation was an arginine substitution for

7

a leucine in codon 596. The corresponding sequence in *ABCG5* is another nonpolar amino acid, glutamine. None of these three missense mutations were identified in 100 alleles from ethnically-matched normolipidemic subjects, which is consistent with these being disease-causing mutations. A common polymorphism (Y54C) with an allele frequency of 23% in control subjects (n=100 alleles) was also identified in *ABCG8*.

Thus, we identified two mutant alleles at the *ABCG8* locus in four of the nine sitosterolemic patients. Four patients had a single mutant allele in *ABCG8* and one patient had a single mutant allele in *ABCG5*. The identification of multiple different *ABCG8* mutations in subjects with sitosterolemia, including nonsense mutations that appear incompatible with protein function, provides strong evidence that sitosterolemia is caused by defects in this gene. It also seems likely that the mutation we found in *ABCG5* causes sitosterolemia, although the identification of additional mutations in this gene will be required to substantiate this deduction. It is possible that some mutations causing sitosterolemia were not detected by SSCP or because they were located in sequences not screened in this study, including regulatory sequences or those required for mRNA processing. Alternatively, it remains possible that mutations in another gene (perhaps a different ABC transporter) within the genomic interval mapped by Patel *et al.* (23) can cause sitosterolemia when present in combination with mutations in *ABCG5* or *ABCG8*.

To determine if *ABCG5* and *ABCG8* are regulated coordinately, we examined the tissue distributions of their mRNAs in humans and mice, and their responses to cholesterol feeding in mice. In humans, liver was the major site of expression of both genes (Fig. 2A). For both *ABCG5* and *ABCG8*, one major transcript predominated but

other mRNAs were visible by RNA blotting. Additional studies will be required to

determine the identity of these transcripts, which presumable result from alternative

splicing of differential polyadenylation. RNAs of the same sizes, as those seen in the liver

were seen in the small intestine on a longer exposure times(data not shown). Transcripts

for both *ABCG5* and *ABCG8* could be PCR-amplified from human intestinal mRNA, but

not from human cholesterol-loaded differentiated monocytes (THP-1 cells) (data not

shown). In mice, *Abcg5* and *Abcg8* were expressed at higher levels in the intestine than

in the liver, although the relative amounts of expression in these two tissues may be

strain- and sex-specific (data not shown). Inasmuch as the expression of these two genes

is regulated by dietary sterols (see below), definitive studies of tissue expression in

humans will require careful control of dietary intake.

If *ABCG5* and *ABCG8* protect against the accumulation of sterols, then their

expression levels would be predicted to increase with cholesterol feeding. To test this

hypothesis, mice were fed a high cholesterol diet (2%) and killed after 1, 7 or 14 days.

The mRNA levels of both genes increased ~2-fold in intestine and much more markedly

in liver within one week of initiation of the high cholesterol diet (Fig. 2B). These

changes were maintained at two weeks (data not shown). As expected, the plasma levels

of cholesterol did not significantly change in the cholesterol-fed mice (from 95 mg/dl

versus 93 mg/dl) since mice rapidly and efficiently convert dietary cholesterol into bile

acids and excrete both cholesterol and bile acids into the bile (36). LXR plays a central

role in this regulated process by increasing the expression of multiple hepatic genes that

promote bile acid synthesis and biliary secretion (11). The ligands for LXR include

hydroxylated sterols that are derived from cholesterol (37,38). Since *ABCG5* is induced

by an LXR agonist, it is possible that dietary sterols induce these genes through LXR.

The aforementioned data indicate that ABCG5 and ABCG8 are putative ABC

half-transporters that may partner to generate a functional protein. The juxtaposition of

these two genes on chromosome 2, the coordinate regulation of their mRNAs in the liver

and intestine with cholesterol feeding, and the observation that mutations in either gene

are associated with sitosterolemia, suggest that these two proteins form a functional

complex that mediates efflux of dietary cholesterol from the intestine, and thus protects

humans from sterol overaccumulation. This protection is especially important in Western

societies that consume high cholesterol diets. Loss of function of these proteins causes

sitosterolemia. It seems possible that subtle defects in these proteins or in their regulation

may underlie the variable responses of individuals to high cholesterol diets.

References and notes

1. A. K. Bhattacharyya, W. E. Connor, *J. Clin. Invest.* **53**, 1033 (1974).

2. I. Bjorkhem, K. M. Boberg, in *The Metabolic and Molecular Bases of Inherited Disease,* C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle, Eds. (McGraw Hill, New York, 1995), pp. 2073 vol. 2, chap. 65 [seventh edition].

3. G. Salen, *et al., J. Lipid Res.* **33**, 945 (1992).

4. T. A. Miettinen, *Eur. J. Clin. Invest.* **10**, 27 (1980).

5. I. Morganroth, R. I. Levy, A. E. McMahon, A. M. Gotto, Jr., *J. Pediatr.* **85**, 639 (1974).

6. G. Salen, *et al., J. Lipid Res.* **26**, 203 (1985).

7. R. E. Gregg, W. E. Connor, D. S. Lin, H. B. Brewer, Jr., *J. Clin. Invest.* **77**, 1864 (1986).

8. R. G. Gould, R. J. Jones, G. V. LeRoy, R. W. Wissler, C. B. Taylor, *Metabolism* **18**, 652 (1969).

9. G. Salen, E. H. Ahrens, Jr., S. M. Grundy, *J. Clin. Invest.* **49**, 952 (1970).

10. G. Salen, *et al., J. Lipid Res.* **26**, 1126 (1985).

11. J. J. Repa, *et al., Science* **289**, 1524 (2000).

12. D. J. Peet, *et al., Cell* **93**, 693 (1998).

13. R. M. Lawn, et al., *J. Clin. Invest.* **104**, 25 (1999).

14. Total RNA was prepared from the liver, intestine and kidney of C57BL/6mice treated with the LXR agonist T091317 (50mg/kg). Duplicate RNA samples were labeled

with two fluorescence dyes and hybridized to mouse GEM1microarrays (performed at

Incyte Genomics, Inc.). Data analysis revealed the relative expression levels of 8734

genes in tissues from the treated versus the untreated mouse tissues.

15. D. T. Sullivan, S. L. Grillo, R. J. Kitos, *J. Exp. Zool.* **188**, 225 (1974).

16. P. M. Bingham, R. Lewis, G. M. Rubin, *Cell* **25**, 693 (1981).

17. C. Higgins, *Annu. Rev. Cell Biol.* **8**, 67 (1992).

18. J. Klucken, et al., *Proc. Natl. Acad. Sci. U. S. A.* **97**, 817 (2000).

19. A.Venkateswatan *et al.*, *J. Biol. Chem.* **12**: 147000 (2000).

20. J. E. Walker, M. Saraste, M. J. Runswick, N. J. Gay, *Embo. J.* **1**, 945 (1982).

21. D. T. Jones, W. R. Taylor, J. M. Thornton, *Biochem.* **33**, 3038 (1994).

22. Chromosomal localization of *ABCG5* was confirmed using primers derived from exon 7 of *ABCG5* to amplify a gene-specific fragment from the TNG panel of radiation hybrids from Stanford Human Genome Center (Research Genetics, Inc). The result was submitted to the RH Server (http://wwwshgc.stanford.edu/cgi-bin), which linked *ABCG5* to SHGC14952, which is between markers D2S177 and D2S119.

23. S. B. Patel, et al., *J. Clin. Invest.* **102**, 1041 (1998).

24. The last 3 exons of *ABCG5* were contained in the GenBank sequence entry AC011242 and were further confirmed by PCR analysis from human genomic DNA. The remaining 10 exon/intron boundaries were determined using PCR and cDNA primers to amplify the exon sequences and the intron/exon boundaries using genomic DNA and cDNA primers followed by sequence analysis.

25. O. Le Saux, et al., *Nat. Genet.* **25**, 223 (2000).

26. The 3'-end of *ABCG5* was located on BAC RP11-489K22, which had been partially sequenced, but no other ABC transporters were identified on this BAC. A BAC end sequences (BES) in the Genome Survey Sequence database that was located on BAC RP11-489K22 was used to search the Celera Human Fragments database. The public and Celera database were used to assemble most of the genomic sequences in the region, resulting in the identification of EST T84531, which shared weak homology with the *drosophila white* gene (11). The GENSCAN Web Server (http://genes.mit.edu/GENSCAN.html) was used to identify additional exons within this gene. The sequence of the ~30 kb region was assembled (excluding three gaps) using the Celera Human Fragments database and mouse ESTs in the public database.

27. S. A. Ikeda, A. Mochizuki, A. H. Sarker, S. Seki, *Biochem. Biophys. Res. Commun.* **273**, 1063 (2000).

28. R. Pollner, C. Schmidt, G. Fischer, K. Kuhn, E. Poschl, *FEBS Lett.* **405**, 31 (1997).

29. M. Orita, Y. Suzuki, T. Sekiya, K. Hayashi, *Genomics* **5**, 874 (1989).

30. H. H. Hobbs, M. S. Brown, J. L. Goldstein, *Hum. Mutat.* **1**, 445 (1992).

31. E. R. Nye, W. H. Sutherland, J. G. Mortimer, H. C. Stringer, *N. Z. Med. J.* **101**, 418 (1988).

32. P. S. Stell, D. L. Sprecher, *Top. Clin. Nutr.* **5**, 63 (1990).

33. G. D. Ewart, D. Cannell, G. B. Cox, A. J. Howells, *J. Biol. Chem.* **269**, 10370-7 (1994).

34. P. O. Kwiterovich, Jr., *et al.*, *Lancet* **1**, 466 (1981).

35. T. H. Beaty, *et al.*, *Am. J. Hum. Genet.* **38**, 492 (1981).

36. D. W. Russell, K. D. Setchell, *Biochem.* **31**, 4737 (1992).

37. B. A. Janowski, *et al.*, *Nature* **383**, 728 (1996).

38. B. A. Janowski *et al*, *Proc. Natl. Acad. Sci.* **96**, 266 (1999).

39. V. Jokinen, *et al.*, *J. Biol. Chem.* **269** 26411 (1994).

Table 1. Molecular defects in nine unrelated individuals with sitosterolemia. Genomic DNA was extracted from cultured fibroblasts or lymphoblasts from the proband or another family member with sitosterolemia (30). All subjects had elevated plasma sitosterol levels (except proband 6 in which plasma sitosterol level were not measured). The age at the time of diagnosis or at the first appearance of xanthomas is provided (when available). The exons and flanking splice site consensus sequences were screened for sequence variations using SSCP and dideoxy-sequencing (20). None of the mutations were found in 100 alleles from normolipidemic controls. The nucleotides are numbered consecutively starting at the A of the initiation codon ATG. Plasma cholesterol levels were obtained from referring physicians or from publications. Abbreviations: y, years; C, fasting plasma cholesterol level; ref., reference; CAD, coronary artery disease; G, Gly; Q, Gln; L, Leu, P, Pro; R, Arg; T, Thr; W, Trp; Y, Tyr.

Subject (age)	Ethnicity	Mutant Alleles	Nucleotide Change	Amino Acid Change(s)	Comments	Ref.
1 (8 y)	German/ Swiss	ABCG8 ABCG8	c.1083G>A c.1083G>A	W361Stop W361Stop	Original case. C=195 mg/dl	1
2 (13 y)	Amish	ABCG8 ABCG8	c.1720G>A c.1720G>A	G574R G574R	13-year-old died of CAD	34
3 (8 mo)	Caucasian American	ABCG8 ABCG8	c.1083G>A c.1974C>G	W361Stop Y658Stop	C fell from 800 to 151 mg/dl on low C diet	
4 (<10 y)	Chinese	ABCG8 ?	c.788G>A ?	R263Q ?	C=556 mg/dl	
5 (5 y)	Caucasian American	ABCG8 ABCG8	c.1083G>A c.1234C>T	W361Stop R412Stop	C fell from 375 to 201 mg/dl on low C diet	5
6 (2 y)	Caucasian American	ABCG8 ?	c.1787T>G ?	L596R ?	C fell from 753 to 106 mg/dl on low C diet	32
7	Mexican-American	ABCG8 ?	1234C>T ?	R412Stop ?	LDL-C fell from 380 to 200 mg/dl	
8 (3.5 y)	Caucasian N. Zealander	ABCG8 ?	c.1083G>A ?	W361Stop ?	C fell from 718 to 127 mg/dl on low C diet	31
9 (<10 y)	Chinese	ABCG5 ?	c.1222C>T ?	R408 Stop ?	C=620 mg/dl	

Figure legends

Fig. 1. Genomic structure (**A**), putative topology (**B**), and predicted amino acid

sequences of *ABCG5* and *ABCG8* (**C**). *ABCG5* and *ABCG8* are located on chromosome

2p21 between markers D2S177 and D2S119. (**A**) *ABCG5* and *ABCG8* are tandemly

arrayed in a head-to-head orientation separated by 374 basepairs. *ABCG5* and *ABCG8*

are both encoded by 13 exons and each spans ~28 kb. (**B**) The mutations detected in

patients with sitosterolemia (Table 1) are indicated on a schematic model of *ABCG5* (left)

and *ABCG8* (right) (**C**) Predicted amino acid sequence of ABCG5 and ABCG8, which

are 651 and 673 residues in length, respectively. Alignment of the inferred amino acid

sequences indicates 28% sequence identity and 61% sequence similarity between *ABCG5*

and *ABCG8*. Both proteins are predicted to contain six transmembrane segments using

the program MEMSAT 2 (21). The putative transmembrane segments of each protein are

indicated by blue (*ABCG5*) or green (*ABCG8*) cylinders (**B**) and lines (**C**). The Walker A

motif and Walker B motifs are highlighted in yellow and pink, respectively. The ABC

signature sequence (C-motif) is indicated in purple.

Fig. 2. Expression of *ABCG5* and *ABCG8* in human tissues (**A**) and the effect of

cholesterol feeding on levels of *ABCG5* and *ABCG8* mRNAs in mouse liver and

intestines (**B**). (**A**) Northern blot analysis of human tissues. The coding sequence of

ABCG5 and *ABCG8* were amplified from liver polyA$^+$ RNA (Clontech) and the

fragments were cloned into the plasmid vector pGEM-T (Promega). The coding region

of the cDNA was amplified and the fragment radiolabeled (Megaprime DNA Labeling System, Amersham) prior to incubation with the blot in Rapid-hyb buffer (1×10^6 cpm/ml) (Amersham). The blot was washed and subjected to autoradiography for 18 h using Kodak X-OMAT-blue film (39). The results were identical when probes generated from the 3' untranslated regions of both cDNAs were used. **(B)** Cholesterol feeding-induces coordinate increases in levels of *ABCG5* and *ABCG8* mRNA. Seven-week-old male mice (129S3/SvImj) were fed powdered chow (Harlan Teklad Rodent Diet) in the absence or presence of cholesterol (2%, w/v). Mice were killed after one or seven days in the light phase of the cycle. Total RNA was isolated using RNA-STAT (TelTest) from the liver and three equal segments of the small intestine (duodenum, jejunum and ileum). The tissue RNAs were pooled from three animals and aliquots (15 μg) used to make duplicate northern blots (30). The mouse cDNAs for *ABCG5* and *ABCG8* were used as probes. Cyclophilin was used as an internal standard. The results were identical when probes generated from the 3' untranslated regions of both cDNAs were used.
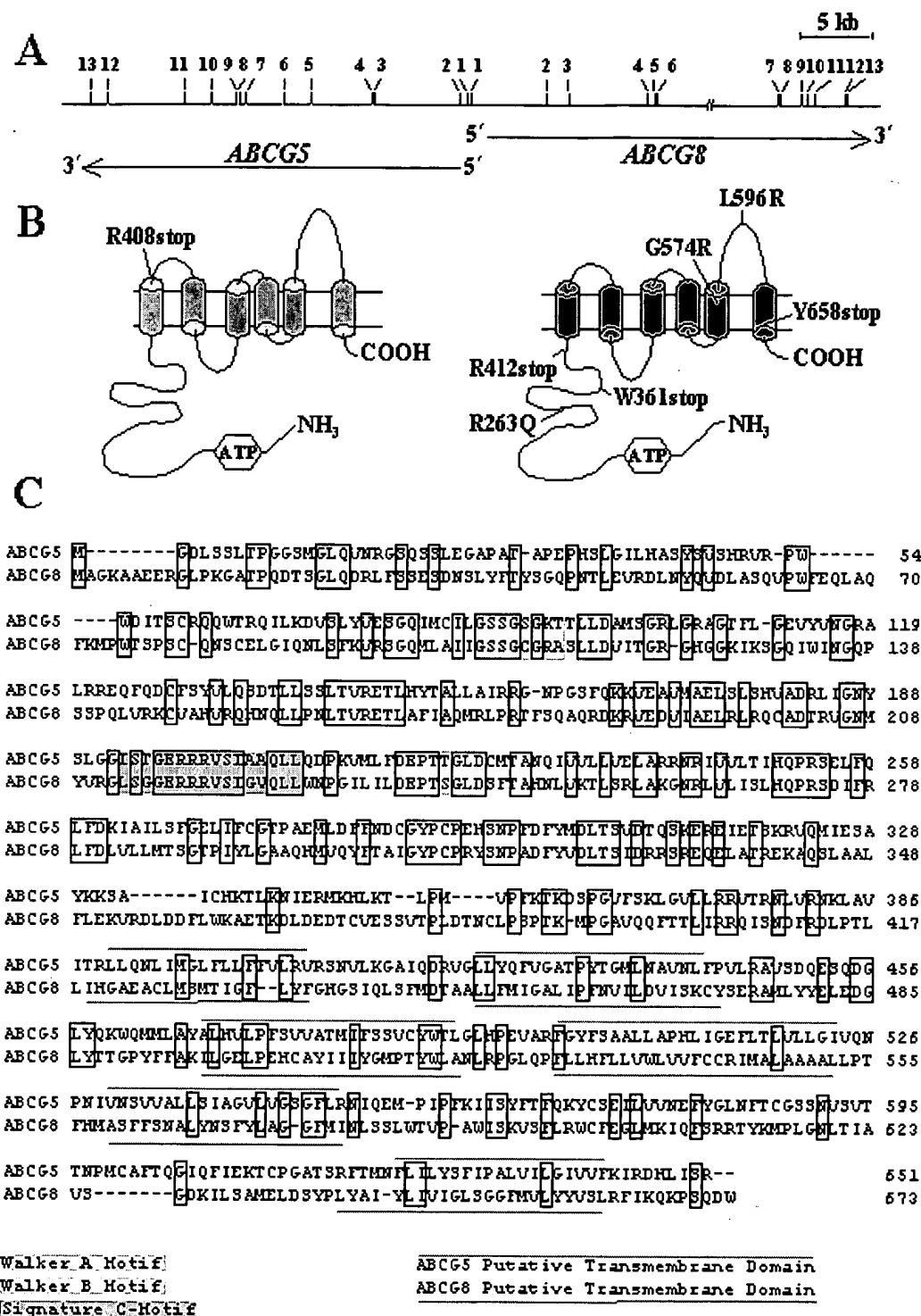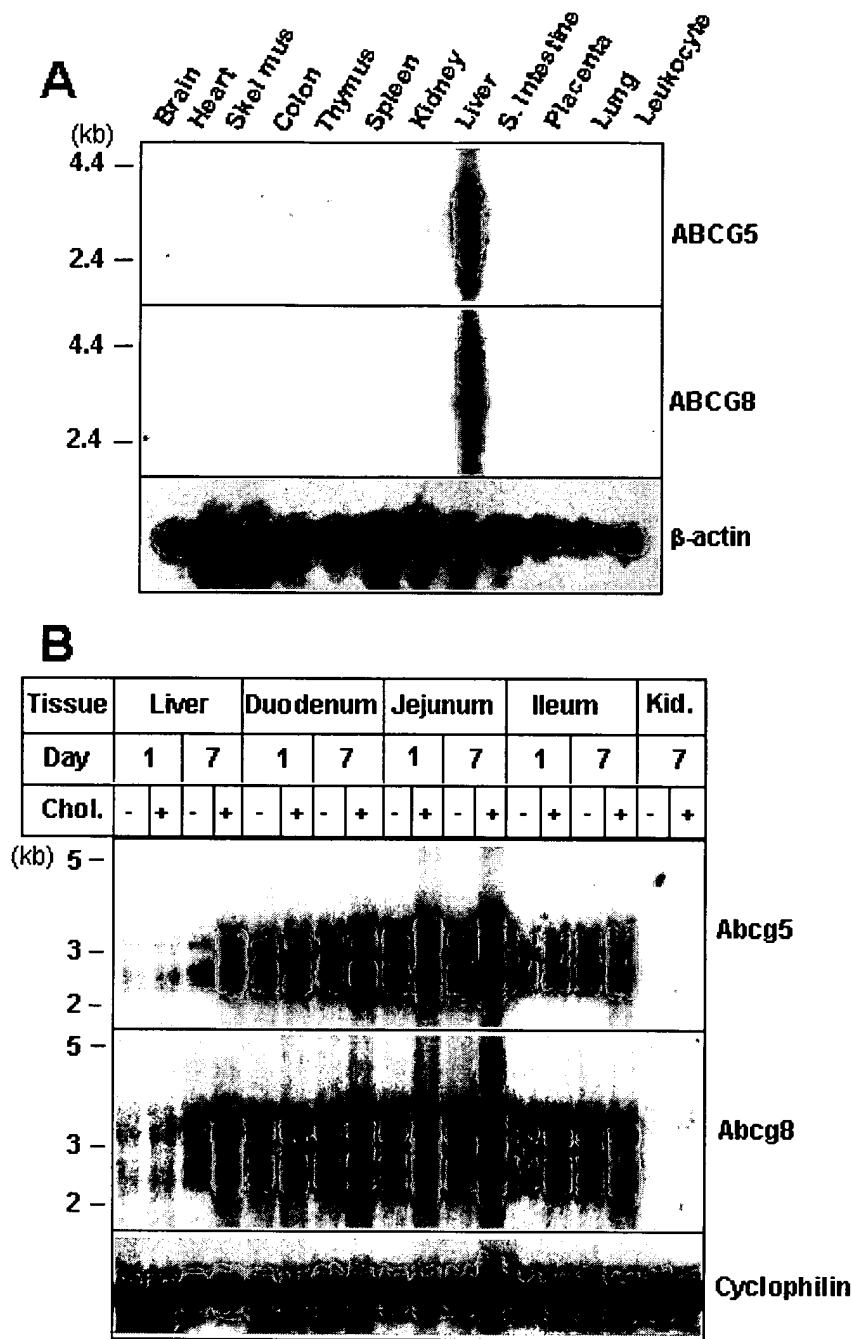
Figure 1.

Figure 2.

From: helen.hobbs@UTSouthwestern.edu
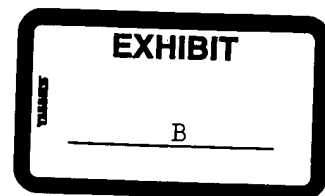**Sent:**
T : "Eugenia G. Wackowski" <egwackowski@townsend.com>
Subject: Re: Patent Application/UTSD:757

Here are the human cDNA sequences from human brown (ABCG5) and ABCG8 (which we call Goldstein) attached. Let me know if these is anything else you need.

Helen Hobbs

>hABCG8
GTGTCCCTGCTCCAGGAAACAGAGTGAAGACACTGGCCCTGGCAGGCAGCAGCTGGGTCTAAGAGAGCTGCAGCCCA
GGGTCACAGACCTGTGGGCCCCATGGCCGGGAAGGCGGCAGAGGAGAGAGGGCTGCCGAAAGGGGCCACTCCCCAGG
ATACCTCGGGCCTCCAGGATAGATTGTTCTCCTCTGAAAGTGACAACAGCCTGTACTTCACCTACAGTGGCCAGCCC
AACACCCTGGAGGTCAGAGACCTCAACTACCAGGTGGACCTGGCCTCTCAGGTCCCTTGGTTTGAGCAGCTGGCTCA
GTTCAAGATGCCCTGGACATCTCCCAGCTGCCAGAATTCTTGTGAGCTGGGCATCCAGAACCTAAGCTTCAAAGTGA
GAAGTGGGCAGATGCTGGCCATCATAGGGAGCTCAGGTTGTGGGAGAGCCTCCTTGCTAGATGTGATCACTGGCCGA
GGTCACGGCGGCAAGATCAAGTCAGGCCAGATCTGGATCAATGGGCAGCCCAGCTCGCCTCAGCTGGTGAGGAAGTG
TGTGGCCCACGTGCGCCAGCACAACCAGCTGCTCCCCAACTTGACTGTGCGAGAGACCTTGGCCTTCATTGCCCAGA
TGCGGCTGCCCAGAACCTTCTCCCAGGCCCAGCGTGACAAAAGGGTGGAGGACGTGATCGCGGAGCTGCGGCTTAGG
CAGTGCGCTGACACCCGCGTGGGCAACATGTACGTGCGGGGGTTGTCGGGGGGTGAGCGCAGGAGAGTCAGCATTGG
GGTGCAGCTCCTGTGGAACCCAGGAATCCTTATTCTCGACGAACCCACCTCTGGGCTCGACAGCTTCACAGCCCACA
ACCTGGTGAAGACCTTGTCCAGGCTGGCCAAAGGCAACCGGCTGGTGCTCATCTCCCTCCACCAGCCTCGCTCTGAC
ATCTTCAGGCTGTTTGATCTGGTCCTCCTGATGACGTCTGGCACCCCCATCTACTTAGGGGCGGCCCAGCACATGGT
CCAGTATTTCACAGCCATCGGCTACCCCTGTCCTCGCTACAGCAATCCTGCTGACTTCTATGTGGACCTGACCAGCA
TTGACAGGCGCAGCAGAGAGCAGGAATTGGCCACCAGGGAGAAGGCTCAGTCACTCGCAGCCCTGTTTCTAGAAAAA
GTGCGTGACTTAGATGACTTTCTATGGAAAGCAGAGACGAAGGATCTTGACGAGGACACCTGTGTGGAAAGCAGCGT
GACCCCACTAGACACCAACTGCCTCCCGAGTCCTACGAAGATGCCTGGGGCGGTGCAGCAGTTTACGACGCTGATCC
GTCGTCAGATTTCCAACGACTTCCGAGACCTGCCCACCCTCCTCATCCATGGGCGGAGGCCTGTCTGATGTCAATG
ACCATCGGCTTCCTCTATTTTGGCCATGGGAGCATCCAGCTCTCCTTCATGGATACAGCCGCCCTCTTGTTCATGAT
CGGTGCTCTCATCCCTTTCAACGTCATTCTGGATGTCATCTCCAAATGTTACTCAGAGAGGGCAATGCTTTACTATG
AACTGGAAGACGGGCTGTACACCACTGGTCCATATTTCTTTGCCAAGATCCTCGGGGAGCTTCCGGAGCACTGTGCC
TACATCATCATCTACGGGATGCCCACCTACTGGCTGGCCAACCTGAGGCCAGGCCTCCAGCCCTTCCTGCTGCACTT
CCTGCTGGTGTGGCTGGTGGTCTTCTGTTGCAGGATTATGGCCCTGGCCGCCGCGGCCCTGCTCCCCACCTTCCACA
TGGCCTCCTTCTTCAGCAATGCCCTCTACAACTCCTTCTACCTCGCCGGGGGCTTCATGATAAACTTGAGCAGCCTG
TGGACAGTGCCCGCGTGGATTTCCAAAGTGTCCTTCCTGCGGTGGTGTTTTGAAGGGCTGATGAAGATTCAGTTCAG
CAGAAGAACTTATAAAATGCCTCTCGGGAACCTCACCATCGCGGTCTCAGGAGATAAAATCCTCAGTGCCATGGAGC
TGGACTCGTACCCTCTCTACGCCATCTACCTCATCGTCATTGGCCTCAGCGGTGGCTTCATGGTCCTGTACTACGTG
TCCTTAAGGTTCATCAAACAGAAACCAAGTCAAGACTGGTGATTCACGCCAGACGTCTGCCCGCTGGTGGGGGACCT
GAGCAGACCCTTCAACTGCACTCCCTCCTCAGGAGCCCCTTCCTGGGGACAGTGAGGACAATGACCCTACAGATGCT
CAGCTACATCCGGCCCAGGGTGCTGCAGTGGCACAGACCAGCCACAGGATGGCAGTAGAATAAAGACAGTCGAAAGG
GATTTCTGCTCACTGGCAGGAGACTGCGATGACTGGGAGAAAACCTGCACTCGGTGGCACCTACAACGTTGCTAATT
TATTTCCTTTTGATATGCATTTATATAGGCAACTCGATATAGGATGGGAGCAAACTAGGAATGAATTGGGTAGCTAG
ACTGTGCAGGAATTGTTGGAACCTGGAGGGAACAATAACAGTAGCTAGCAGATTTGGCTTCATCTTCCAGGGGCCCC
ACACTCCGTGGTGAGCCACCATCAATACAGAAAGTGACCTAAGATGTACCAGCAAGATGCCATCCCTTCTTTTGTG
TGGGGTCATGGGCTCCAAAAGCCAACGTGAACAATTAAAAATGTATTGAGC

>hABCG5
GTCAGGTGGAGCAGGCAGGGCAGTCTGCCACGGGCTCCCCAACTGAAGCCACTCTGGGGAGGGTCCGGCCACCAGAA
AATTTGCCCAGCTTTGCTGCCTGTTGGCCATGGGTGACCTCTCATCTTTGACCCCCGGAGGGTCCATGGGTCTCCAA
GTAAACAGAGGCTCCCAGAGCTCCCTGGAGGGGGCTCCTGCCACCGCCCCGGAGCCTCACAGCCTGGGCATCCTCCA
TGCCTCCTACAGCGTCAGCCACCGCGTGAGGCCCTGGTGGACATCACATCTTGCCGGCAGCAGTGGACCAGGCAGA
TCCTCAAAGATGTCTCCTTGTACGTGGAGAGCGGGCAGATCATGTGCATCCTAGGAAGCTCAGGCTCCGGGAAAACC
ACGCTGCTGGACGCCATGTCCGGGAGGCTGGGGCGCGCGGGGACCTTCCTGGGGGAGGTGTATGTGAACGGCCGGGC
GCTGCGCCGGGAGCAGTTCCAGGACTGCTTCTCCTACGTCCTGCAGAGCGACACCCTGCTGAGCAGCCTCACCGTGC
GCGAGACGCTGCACTACACCGCGCTGCTGGCCATCCGCCGCGGCAATCCCGGCTCCTTCCAGAAGAAGGTGGAGGCC
GTCATGGCAGAGCTGAGTCTGAGCCATGTGGCAGACCGACTGATTGGCAACTACAGCTTGGGGGGCATTTCCACGGG
TGAGCGGCGCCGGGTCTCCATCGCAGCCCAGCTGCTCCAGGATCCTAAGGTCATGCTGTTTGATGAGCCAACCACAG
GCCTGGACTGCATGACTGCTAATCAGATTGTCGTCCTCCTGGTGGAACTGGCTCGCAGGAACCGAATTGTGGTTCTC
ACCATTCACCAGCCCCGTTCTGAGCTTTTTCAGCTCTTTGACAAAATTGCCATCCTGAGCTTCGGAGAGCTGATTTT
CTGTGGCACGCCAGCGGAAATGCTTGATTTCTTCAATGACTGCGGTTACCCTTGTCCTGAACATTCAAACCCTTTTG
ACTTCTATATGGACCTGACGTCAGTGGATACCCAAAGCAAGGAACGGGAAATAGAAACCTCCAAGAGAGTCCAGATG
ATAGAATCTGCCTACAAGAAATCAGCAATTTGTCATAAAACTTTGAAGAATATTGAAAGAATGAAACACCTGAAAAC
GTTACCAATGGTTCCTTTCAAAACCAAAGATTCTCCTGGAGTTTTCTCTAAACTGGGTGTTCTCCTGAGGAGAGTGA
CAAGAAACTTGGTGAGAAATAAGCTGGCAGTGATTACGCGTCTCCTTCAGAATCTGATCATGGGTTTGTTCCTCCTT
TTCTTCGTTCTGCGGGTCCGAAGCAATGTGCTAAAGGGTGCTATCCAGGACCGCGTAGGTCTCCTTTACCAGTTTGT
GGGCGCCACCCCGTACACAGGCATGCTGAACGCTGTGAATCTGTTTCCCGTGCTGCGAGCTGTCAGCGACCAGGAGA

GTCAGGACGGCCTCTACCAGAAGTGGCAGATGATGCTGGCCTATGCACTGCACGTCCTCCCCTTCAGCGTTGTTGCC
ACCATGATTTTCAGCAGTGTGTGCTACTGGACGCTGGGCTTACATCCTGAGGTTGCCCGATTTGGATATTTTTCTGC
TGCTCTCTTGGCCCCCCACTTAATTGGTGAATTTCTAACTCTTGTGCTACTTGGTATCGTCCAAAATCCAAATATAG
TCAACAGTGTAGTGGCTCTGCTGTCCATTGCGGGGGTGCTTGTTGGATCTGGATTCCTCAGAAACATACAAGAAATG
CCCATTCCTTTTAAAATCATCAGTTATTTTACATTCCAAAAATATTGCAGTGAGATTCTTGTAGTCAATGAGTTCTA
CGGACTGAATTTCACTTGTGGCAGCTCAAATGTTTCTGTGACAACTAATCCAATGTGTGCCTTCACTCAAGGAATTC
AATTCATTGAGAAAACCTGCCCAGGTGCAACATCTAGATTCACAATGAACTTTCTGATTTTGTATTCATTTATTCCA
GCTCTTGTCATCCTAGGAATAGTTGTTTTCAAAATAAGGGATCATCTCATTAGĆAGGTAGTGAAAGCCATGGCTGGG
AAAATGGAAGTGAAGCTGCCGACTGTGCATGACTGCTCTGAACGTCTGAAATGAGAGTGCCATGTATTTCTTTCTTG
ACAGGACATCTCAAGTCTTTTAACCATTAAGACTCCATTTGTGCCTCTTGGATCCAAGCAGGCCTTGAATGCAATGG
AAGTGGTTTATAGTCCCTTGCTCTTACAACTTGCAGGGACATGTGGTTATTTGGAAATTGTGACTGAGCGGACCCAA
GAATGTAAATAATATTCATAAACCTATGGG